Loss of Haem in Rat Liver Caused by the Porphyrogenic Agent 2-Allyl-2-isopropylacetamide

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1. The effect of a single dose of 2-allyl-2-isopropylacetamide on the cytochrome *P*-450 concentration in rat liver microsomal fraction was studied. The drug caused a rapid loss of cytochrome *P*-450 followed by a gradual increase to above the normal concentration. 2. The loss of cytochrome *P*-450 was accompanied by a loss of microsomal haem and by a brown-green discoloration of the microsomal fraction suggesting that a change in the chemical constitution of the lost haem had taken place. Direct evidence for this was obtained by prelabelling the liver haems with radioactive 5-aminolaevulate: the drug caused a loss of radioactivity from the haem with an increase of radioactivity in a fraction containing certain unidentified green pigments. 3. Evidence was obtained by a dual-isotopic procedure that rapidly turning-over haem(s) may be preferentially affected. 4. The loss of cytochrome *P*-450 as well as the loss of microsomal haem and the discoloration of the microsomal fraction were more intense in animals pretreated with phenobarbitone and were much less evident when compound SKF 525-A (2-diethylaminoethyl 3,3-diphenylpropylacetate) was given before 2-allyl-2-isopropylacetamide, suggesting that the activity of the drug-metabolizing enzymes may be involved in these effects. 5. The relevance of the destruction of liver haem to the increased activity of 5-aminolaevulate synthetase caused by 2-allyl-2-isopropylacetamide is discussed.

Several chemically unrelated lipid-soluble drugs stimulate the hepatic formation of porphyrins in the experimental animal and in liver cells cultured *in vitro* (De Matteis, 1967, 1971), and in both systems enhance the activity of 5-aminolaevulate synthetase, the rate-limiting enzyme in the biosynthetic pathway of porphyrins and haem (Granick & Urata, 1963; Granick, 1966). This is thought to result from an interference by the drugs with the feedback control exercised by haem at the level of the enzyme (Granick, 1966), although the exact mechanism of this effect is not known.

It has been reported (De Matteis, 1970) that one of these drugs, 2-allyl-2-isopropylacetamide, causes a rapid loss of cytochrome *P*-450 and haem from rat liver microsomal fraction coincidental with the rise in activity of 5-aminolaevulate synthetase. Preliminary evidence has also been presented indicating that the lost haem undergoes a change in chemical constitution, probably to certain ill-defined green pigments (referred to below as 'green pigments') already described in the liver of animals with experimental porphyria (Schwartz & Ikeda, 1955).

The present paper provides further information on the destruction of liver haem caused by 2-allyl-2-isopropylacetamide. The significance of this loss of haem in relation to the induction of 5-aminolaevulate synthetase caused by the drug is discussed.

MATERIALS AND METHODS

Treatment of animals. Male albino rats (150-180 g) of the Porton strain were kept in experimental cages that had been designed to prevent coprophagy. They were starved for 24 h before being injected with 2-allyl-2-isopropylacetamide (20 mg/ml of 0.9% NaCl) in the loose subcutaneous tissue of the neck and kept starved until they were killed by decapitation. Control animals were injected with 0.9% NaCl alone. Finely powdered phenylbutazone (4-*n*-butyl-1,2-diphenyl-3,5-dioxypyrazolidine) was suspended in arachis oil (15 mg/ml) and given by stomach tube.

In some experiments the rats were treated with phenobarbitone (5-ethyl-5-phenylbarbiturate) or DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] or compound SKF 525-A (2-diethylaminoethyl 3,3-diphenylpropylacetate) before the administration of 2-allyl-2-isopropylacetamide. Phenobarbitone was given intraperitoneally.

in two doses, 6h apart, of 50mg/kg body wt. 2 days before and in a single dose of 80mg/kg body wt. 1 day before the 2-allyl-2-isopropylacetamide. Treatment with DDT involved a single intraperitoneal injection of 75mg/kg body wt. (in arachis oil) 72h before the 2-allyl-2-isopropylacetamide. Compound SKF 525-A was given intraperitoneally (40mg/kg body wt. dissolved in 0.9% NaCl) 45 min before the 2-allyl-2-isopropylacetamide.

In the labelling experiments 5-amino[4- 14 C]laevulate (1.5 μ Ci/rat) and [G- 3 H]5-aminolaevulate (8.7 μ Ci/rat) were injected intraperitoneally 25h and 2h respectively before the administration of 2-allyl-2-isopropylacetamide. Each rat usually received both the 3 H- and the 14 C-labelled precursor. Other animals were given 50 μ Ci of [59 Fe]ferrous ascorbate/kg body wt. intraperitoneally 2h before the 2-allyl-2-isopropylacetamide. The labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and had the following specific radioactivities: 5-amino[4- 14 C]laevulate, 19–53 mCi/mmol; [G- 3 H]5-aminolaevulate, 300 mCi/mmol; [59 Fe]ferrous ascorbate, 12 mCi/mg of Fe (at the time of issue).

Isolation of liver mitochondria and microsomal fraction. Liver homogenates (10%, w/v) were prepared in ice-cold 0.25M-sucrose as described by Bond & De Matteis (1969). The homogenate (30ml) was centrifuged at 850g for 10 min and the supernatant spun again at 6500g for 15 min to obtain a mitochondrial pellet. The mitochondria were washed once with 30 ml of 0.25M-sucrose and resuspended in 8–10 ml of 0.25M-sucrose. The microsomal fraction was obtained by spinning the supernatant of a 9000g (20 min) centrifugation at 105000g for 1h. The microsomal pellet was then washed once with 1.15% (w/v) KCl and suspended in 0.1M-sodium-potassium phosphate buffer, pH 7.4, so that 1 ml contained the microsomal fraction from 20–40 mg wet wt. of liver. When haem was to be determined, the microsomal pellet was suspended in the phosphate buffer so that 1 ml contained the microsomal fraction from 250 mg wet wt. of liver.

Assay of mitochondrial 5-aminolaevulate synthetase activity. Mitochondria (7–15 mg of protein) were incubated for 30 min in 25 ml conical flasks at 38°C in air with shaking (60 cycles/min). Each flask contained in a total volume of 2.6 ml the following components, with total amounts present in parentheses: glycine (200 μ mol), EDTA (20 μ mol), MgCl_2 (40 μ mol), tris-HCl buffer (100 μ mol), sodium-potassium phosphate buffer (50 μ mol), sodium citrate (100 μ mol), ATP (2 μ mol) and sucrose (475 μ mol); the pH of the complete mixture was adjusted to 7.3 (at 20°C) with NaOH. The reaction was stopped by addition of 0.25 ml of 50% (w/v) trichloroacetic acid and the supernatant analysed for 5-aminolaevulate. Under these conditions the formation of 5-aminolaevulate was linear with protein concentration up to at least 30 mg of mitochondrial protein. A blank and a standard containing 60 nmol of 5-aminolaevulate were prepared for each experiment. A 'zero-time' sample was obtained for each mitochondrial preparation by adding the trichloroacetic acid to the complete mixture before incubation.

Colorimetric determination of 5-aminolaevulate. The method used for the determination of 5-aminolaevulate is based on its property of condensation with acetylacetone to yield a pyrrole (Mauzerall & Granick, 1956)

that possesses a free carboxyl group and that, unlike the pyrrole similarly produced from aminoacetone, does not partition into ether at alkaline pH (Gibson, Laver & Neuberger, 1958; Granick, 1966). The supernatant of the trichloroacetic acid deproteinization (2 ml) was pipetted into a glass-stoppered test tube: 1.2 ml of a 1:2 (v/v) mixture of 1M-NaOH and 1M-sodium acetate buffer, pH 4.6, was added, followed by 40 μ l of acetylacetone. The tube was tightly stoppered, then heated in boiling water for 10 min. After the mixture had cooled, 0.15 ml of freshly prepared aqueous 66.0 mM-N-ethylmaleimide was added, followed 30 min later by approx. 0.07 ml of 10M-NaOH to adjust the pH to 8.0. This neutralizes any free thiol groups, which might interfere with the intensity and stability of the Ehrlich reaction (Rimington, Krol & Tooth, 1956). The solution was then transferred to a 20 ml separating funnel and shaken with 3 ml of equilibrated ether (obtained by shaking the organic solvent with an equal volume of aqueous solution of pH 8.0 containing trichloroacetic acid, tris-HCl buffer, phosphate buffer, acetate buffer and NaOH in the same concentrations present in the deproteinized samples analysed for 5-aminolaevulate). After complete separation of the two phases, 3 ml of the water phase was mixed with 0.2 ml of acetic acid to bring the pH back below 5. All steps at pH 8 were carried through as quickly as possible: failure to do this occasionally resulted in high blank values. Then 3.2 ml of modified Ehrlich reagent, 2M with respect to perchloric acid (Mauzerall & Granick, 1956), was added. Readings were taken at 556 nm 25 min after the addition of the Ehrlich reagent, and an extinction coefficient of 56.8 $\text{mm}^{-1} \cdot \text{cm}^{-1}$ was used to calculate the concentration of 5-aminolaevulate. This value was obtained experimentally by subjecting known amounts of the amino ketone to the whole procedure. When 5-aminolaevulate and aminoacetone were mixed together in different proportions in the original sample and the complete procedure described above was followed, 92–98% of the 5-aminolaevulate was recovered, but less than 6% of the original aminoacetone.

No attempt was made to identify as 5-aminolaevulate the compound produced on incubation and responsible for the colour reaction with the Ehrlich reagent. However, in one experiment the amounts of 5-aminolaevulate synthesized *in vitro* by liver homogenates from rats treated with 2-allyl-2-isopropylacetamide and from their controls were determined by the method described above and also by the ion-exchange-column method of Marver, Tschudy, Perlroth, Collins & Hunter (1966). Identical results were obtained with the two methods.

Determination of microsomal cytochromes and protohaem. The microsomal cytochromes P-450 and b_5 were determined as described by Bond & De Matteis (1969) with a Unicam SP.800 recording spectrophotometer. The total protohaem content of the washed microsomal fraction was determined as follows: the microsomal fraction was mixed with an equal volume of 1% (w/v) sodium deoxycholate, and after 10–15 min the clarified microsomal suspension was added to an NaOH-pyridine mixture and the haem content was determined at once. The difference spectra between the $\text{Na}_2\text{S}_2\text{O}_4$ -reduced and $\text{K}_3\text{Fe}(\text{CN})_6$ -oxidized haem solutions in 0.15M-NaOH and 25% (v/v) pyridine were recorded and an extinction coefficient of 32.4 $\text{mm}^{-1} \cdot \text{cm}^{-1}$ for the difference in absorption at

557nm and 575nm (Omura & Sato, 1964) was used to calculate the concentration of haem.

In a previous study (Bond & De Matteis, 1969) the total microsomal haem recovered from both normal and phenobarbitone-treated rats was less than the amount expected from the combined concentrations of cytochromes *P*-450 and *b*₅. It has now been found that the low recovery was largely due to a spontaneous reduction of the microsomal haem (even in the presence of air) in the 'oxidized' sample and that the addition of 150–300nmol of $K_3Fe(CN)_6$ (Falk, 1964) to this sample resulted in a much better recovery.

Other enzymic and analytical methods. Glucose 6-phosphatase activity was assayed by the method of Swanson (1955). Protein was determined by Aldridge's (1962) modification of the method of Robinson & Hogden (1940), with crystalline bovine albumin as the standard. Isolated haem was determined as the pyridine haemochrome by the method of Rimington (1942).

Isolation of haem and 'green pigments'. A 5ml portion of a 10% liver homogenate was mixed with horse erythrocyte lysate (to provide 20mg of carrier haem) and treated with 20vol of ethyl acetate-acetic acid mixture (4:1, v/v). After filtration the ethyl acetate solution was successively shaken with one-quarter of its volume of 3% (w/v) sodium acetate (three times) and once with water. After addition of 0.5vol. of light petroleum (b.p. 40–60°C) to the ethyl acetate phase the 'green pigments' were extracted from the organic phase with 0.03 vol. of 7.5M-HCl (15–20 times) as described by Schwartz, Berg, Bossenmaier & Dinsmore (1960). The 7.5M-HCl extracts were washed twice with ethyl acetate to remove traces of haemin. The 'green pigments' were then transferred to diethyl ether: the pooled 7.5M-HCl extracts were adjusted to pH4 by the addition of solid sodium acetate and shaken twice with 50ml of peroxide-free diethyl ether. The ether extracts were pooled, washed with water and evaporated to dryness in a rotary evaporator.

After extraction with 7.5M-HCl the ethyl acetate-light petroleum solution of haem was washed once with a little water and evaporated to dryness. Haemin was crystallized either by the method of Wildy, Nizet & Benson (1961) or by that of Labbe & Nishida (1957).

Liquid-scintillation counting of radioactivity. Portions (0.5ml) of total liver homogenate were placed in counting vials, and solubilized by adding 0.5ml of Soluene 100 to each vial and allowing the vials to stand at room temperature overnight. The 'green pigments' were also dissolved in Soluene 100 and portions were taken for counting of radioactivity.

The haemin was dissolved in pyridine, the solution filtered, and a small portion taken for determination of haem as the pyridine haemochrome. Another portion (equivalent to 80–120µg of haem) was taken for counting of radioactivity: after the pyridine solution had been evaporated to dryness, the haemin was dissolved in 0.5ml of Soluene 100.

Portions (15ml) of a scintillator fluid [a 2:1 (v/v) toluene-ethylCellosolve mixture containing 0.36% (w/v) of 2,5-diphenyloxazole and 0.01% (w/v) of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] were added to the vials containing haemin, 'green pigments' or solubilized tissue for counting of radioactivity.

All measurements of radioactivity were carried out in a

Packard Tri-Carb liquid-scintillation spectrometer and corrected for quenching by the use of an internal standard. When both 3H and ^{14}C were present in the same sample a dual-channel counting procedure was followed and the radioactivities of the two isotopes were calculated with the help of a computer program kindly devised by Mrs P. Corney for the Olivetti Programma 101 desk computer: when the radioactivity of haem was measured under these conditions, the efficiency of counting was 14% for ^{14}C and 4.5% for 3H .

Counting of the radioactivity of samples labelled with ^{59}Fe . This was carried out in a Packard Autogamma scintillation spectrometer: the samples of haemin and 'green pigments' were prepared essentially as described above for liquid-scintillation counting except that the radioactivity of the 'green pigments' was counted in Soluene 100 solution and that of haemin in pyridine solution.

Calculation of results. Results are expressed as the arithmetic means $\pm$ S.E.M. or S.D. and the means were compared by Student's *t* test as modified by Fisher (1934) for small samples. To obtain the value of *P*, a degree of freedom $N_1 + N_2 - 2$ was used throughout.

Source of special chemicals. Cycloheximide and 5-aminolaevulate hydrochloride were obtained from Sigma Chemical (London) Co. Ltd., London S.W.6, U.K., and glucose 6-phosphate, NADP⁺, and glucose 6-phosphate dehydrogenase from Boehringer Corp. (London) Ltd., London W.5, U.K. Carbon monoxide (C.P. grade, at least 99.5% pure) was obtained from the British Oxygen Co. Ltd., London S.W.19, U.K. The solubilizing agent Soluene 100 was obtained from Packard Instrument Ltd., Wembley, Middx., U.K.

RESULTS AND DISCUSSION

Effect of 2-allyl-2-isopropylacetamide on the cytochrome *P*-450 concentration in rat liver microsomal fraction. The effect of 2-allyl-2-isopropylacetamide on the cytochrome *P*-450 concentration in rat liver microsomal fraction was found to depend on the time elapsed between administration of the drug and killing of the animal. When starved rats were given a single dose of 2-allyl-2-isopropylacetamide there was a rapid decrease in the cytochrome *P*-450 concentration in the liver microsomal fraction, followed from about 10h by a gradual increase up to values higher than that observed at the time of dosing (Fig. 1). In contrast, a single dose of phenylbutazone, a drug that does not produce porphyria, did not result in any early loss of the pigment (Fig. 1).

The concentrations of cytochromes *P*-450 and *b*₅ and of proteins in rat liver microsomal fraction at 5h and 48h after administration of 2-allyl-2-isopropylacetamide are shown in Table 1. Both the initial decrease and the later increase in cytochrome *P*-450 concentration were equally evident whether the concentration was referred to the total liver/100g body wt., to unit wet wt. of liver or to unit wt. of microsomal protein. No significant changes

were noted at 5h in either cytochrome b_5 or microsomal protein content of the total liver, whereas at 48h both were increased together with that of cytochrome $P-450$.

These findings suggested that 2-allyl-2-iso-

Fig. 1. Effect of a single dose of 2-allyl-2-isopropylacetamide (○) or of phenylbutazone (■) on the cytochrome $P-450$ concentration in liver microsomal fraction of the rat at different times after dosing. 2-Allyl-2-isopropylacetamide (400 mg/kg body wt.) dissolved in saline was given subcutaneously and phenylbutazone (150 mg/kg body wt.) suspended in arachis oil was given orally. Rats were starved for 24h before they were dosed and were kept starved until they were killed. Results are given as means $\pm$ S.D. (vertical bars) of at least four observations.

propylacetamide possessed two different actions: one shared by many drugs, e.g. phenylbutazone, that leads to increased concentrations of cytochrome $P-450$ as well as of cytochrome b_5 and microsomal protein (F. De Matteis & A. Gibbs, unpublished work) and that is most obvious several hours after dosing, and a second one, not possessed by drugs like phenylbutazone, that concerns cytochrome $P-450$ more specifically and leads to an early loss of this cytochrome.

Demonstration that the decrease in cytochrome $P-450$ concentration caused by 2-allyl-2-isopropylacetamide is due to loss of existing pigment. There are at least three mechanisms that might account for the decrease in cytochrome $P-450$ concentrations caused by 2-allyl-2-isopropylacetamide, namely inhibition of haem and/or cytochrome formation, loss of existing cytochrome or interference by the drug with the development of the characteristic spectrum with carbon monoxide. To distinguish between these possibilities, the decrease in the cytochrome $P-450$ concentration after a single dose of 2-allyl-2-isopropylacetamide was studied in rats that, at the time of administration of the drug, had greatly increased concentrations of microsomal cytochrome $P-450$ in their livers because they had been pretreated with phenobarbitone. The rate of the decrease caused by 2-allyl-2-isopropylacetamide in these animals was compared with that obtained after complete or nearly complete inhibition of liver protein synthesis by cycloheximide (Fig. 2). Cycloheximide caused a very slow decrease in cytochrome $P-450$ concentration: the rate observed was compatible with a half-life of more than 12h (assuming that cycloheximide does not interfere with the degradation of the cytochrome). In contrast, after the administration of 2-allyl-2-isopropylacetamide the cytochrome $P-450$ con-

Table 1. Effect of a single dose of 2-allyl-2-isopropylacetamide on the size of the liver and on the concentrations of cytochromes and protein in liver microsomal fraction of the rat 5h and 48h after treatment

Rats were starved for 24h before being injected with 2-allyl-2-isopropylacetamide or saline and kept starved until they were killed. 2-Allyl-2-isopropylacetamide was given in a single dose of 400 mg/kg body wt. The results are expressed as the means $\pm$ S.E.M. of four observations. Probabilities of difference from corresponding saline-treated controls are indicated thus: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Treatment and time of killing (after injection)	Loss of body wt. due to treatment (%)	Liver wet wt. (g/100g body wt.)	Microsomal cytochrome $P-450$ (nmol/total liver per 100g body wt.)	Microsomal cytochrome b_5 (nmol/total liver per 100g body wt.)	Microsomal protein (mg/total liver per 100g body wt.)
Saline, 5h	—	3.2 ± 0.08	72 ± 6	45 ± 2	61 ± 2
2-Allyl-2-isopropylacetamide, 5h	—	$3.5 \pm 0.08^*$	$33 \pm 2^{**}$	40 ± 2	58 ± 3
Saline, 48h	10.7 ± 0.34	2.9 ± 0.06	82 ± 4	54 ± 5	64 ± 5
2-Allyl-2-isopropylacetamide, 48h	12.2 ± 0.75	$4.0 \pm 0.11^{***}$	$152 \pm 12^{**}$	$76 \pm 3^{**}$	$85 \pm 3^*$

centration was halved in less than 1 h: this very rapid rate of decrease strongly suggested that the drug decreased the cytochrome *P*-450 concentration either by inhibiting the appearance of the characteristic spectrum with carbon monoxide or by causing a loss of the existing pigment. The fall in cytochrome *P*-450 concentration caused by 2-allyl-2-isopropylacetamide was accompanied by a loss of microsomal haem (Table 2), demonstrating conclusively that there was a loss of the existing cytochrome; it was also accompanied by a charac-

teristic brown-green discoloration of the whole liver, that was particularly intense in the microsomal fraction. More direct evidence is presented below indicating that the loss of haem is due to a change in its chemical constitution and conversion into certain unidentified 'green pigments'.

In contrast with the marked loss of microsomal cytochrome *P*-450 and haem observed after the administration of 2-allyl-2-isopropylacetamide to phenobarbitone-treated rats, the cytochrome *b*₅ and protein content of the microsomal fraction and the glucose 6-phosphatase activity of the liver were only slightly decreased when referred to unit wet wt. of liver; they did not change when related to the whole liver/100 g body wt. (Table 3). This finding indicated that the loss of cytochrome *P*-450 caused by 2-allyl-2-isopropylacetamide was not produced through a general effect on the endoplasmic reticulum leading to a loss of all components of the microsomal fraction, but was more likely to result from a more specific (or at least preferential) effect on cytochrome *P*-450.

Activity of the drug-metabolizing enzyme system of rat liver microsomal fraction and destruction of cytochrome P-450 by 2-allyl-2-isopropylacetamide. The decline in cytochrome *P*-450 concentration caused by 2-allyl-2-isopropylacetamide *in vivo* was found to be affected by treatments known to alter the activity of the microsomal drug-metabolizing enzymes (Table 4). Pretreatment of rats with phenobarbitone or DDT, which stimulate drug metabolism in the liver microsomal fraction, greatly increased the loss of cytochrome *P*-450 caused by 2-allyl-2-isopropylacetamide, whereas compound SKF-525A, an inhibitor of drug-metabolizing enzymes (Fouts & Brodie, 1955), decreased the loss. Compound SKF-525A also partially prevented the increased loss of cytochrome *P*-450 observed after phenobarbitone pretreatment (Table 4).

The intensity of brown-green discoloration of the liver and of the microsomal fraction that accompanied the fall in cytochrome *P*-450 concentration

Fig. 2. Effect of a single subcutaneous injection of 2-allyl-2-isopropylacetamide (●, 400 mg/kg body wt.) or of a single intraperitoneal injection of cycloheximide (Δ, 40 mg/kg body wt; ▲, 4 mg/kg body wt.) on the concentrations of cytochrome *P*-450 in liver microsomal fraction of phenobarbitone-induced rats. Each point represents the mean $\pm$ S.E.M. of the logarithm of the value observed. The numbers of observations are given in parentheses.

Table 2. Cytochrome and protohaem content of liver microsomal fraction of the rat after the administration of 2-allyl-2-isopropylacetamide

Phenobarbitone-treated rats (six animals per group) were injected subcutaneously with saline or 2-allyl-2-isopropylacetamide (400 mg/kg body wt.) and killed 1 h later. The results are expressed as means $\pm$ S.E.M. Probabilities of difference from corresponding saline-treated controls are indicated thus: *** $P < 0.001$.

Microsomal component	Microsomal content (nmol/g wet wt. of liver)	
	Saline	2-Allyl-2-isopropylacetamide
Cytochrome <i>P</i> -450	64.3 $\pm$ 4	17.8 $\pm$ 0.7***
Cytochrome <i>b</i> ₅	17.9 $\pm$ 0.8	16.5 $\pm$ 0.7
Cytochromes <i>P</i> -450 + <i>b</i> ₅	82.2	34.3
Total protohaem	74.9 $\pm$ 7.6	34.3 $\pm$ 1.8***

Table 3. *Effect of a single dose of 2-allyl-2-isopropylacetamide on the concentrations of cytochromes and protein in liver microsomal fraction and on the glucose 6-phosphatase activity of liver homogenate of the rat*

Phenobarbitone-treated rats (four per group) were injected subcutaneously with saline or 2-allyl-2-isopropylacetamide (400 mg/kg body wt.) and killed 5 h later. The results are expressed as means $\pm$ s.e.m. Probabilities of difference from corresponding saline-treated controls are indicated thus: ** $P < 0.01$; *** $P < 0.001$.

Treatment ...	Microsomal content or homogenate activity		Loss due to 2-allyl-2-isopropylacetamide (%)
	Saline	2-Allyl-2-isopropylacetamide	
Liver wt. (g/100 g body wt.)	4.1 $\pm$ 0.1	4.38 $\pm$ 0.1	—
Microsomal cytochrome <i>P</i> -450 (nmol/g wet wt. of liver)	53.6 $\pm$ 4.0	11.5 $\pm$ 0.45***	78.6
Microsomal cytochrome <i>b</i> ₅ (nmol/g wet wt. of liver)	15.8 $\pm$ 0.1	13.7 $\pm$ 0.5**	13.3
Microsomal protein (mg/g wet wt. of liver)	23.6 $\pm$ 0.4	22.1 $\pm$ 0.5	6.4
Glucose 6-phosphatase (μ mol of P _i liberated/30 min per g wet wt. of liver)	206.5 $\pm$ 2.5	194.5 $\pm$ 4.6	5.8

after the administration of 2-allyl-2-isopropylacetamide appeared to be related to the rate and extent of cytochrome *P*-450 loss; it was much more pronounced in animals pretreated with phenobarbitone or DDT than in normal animals, and much less apparent in rats given compound SKF-525A before 2-allyl-2-isopropylacetamide (Table 4). The same correlation was found for the loss of total microsomal haem.

A preliminary study of the kinetics of cytochrome *P*-450 loss after the administration of 2-allyl-2-isopropylacetamide suggests the existence of two phases in the decrease of the cytochrome concentration: an initial rapid phase followed by a slow one (Fig. 3). The increased loss of cytochrome *P*-450 observed in animals pretreated with phenobarbitone (as compared with that seen in normal animals) appears to concern mostly, if not entirely, the rapid phase (Fig. 3). It is therefore possible that pretreatment with phenobarbitone or DDT stimulates the destruction of cytochrome *P*-450 by 2-allyl-2-isopropylacetamide by making more of the cytochrome available for destruction, perhaps by increasing the concentration in the microsomal fraction of a portion of the cytochrome *P*-450 that is susceptible to rapid destruction. The protective effect of compound SKF 525-A might be explained by a competition of this drug with 2-allyl-2-isopropylacetamide for sites in the microsomal fraction where the latter compound has to become bound in order to produce its effect on cytochrome *P*-450.

An alternative (or additional) mechanism is, however, possible. Kaufman, Swanson & Marver (1970) have shown that pretreatment of rats with phenobarbitone stimulates the metabolism of 2-allyl-2-isopropylacetamide by the liver. If a metabolite of 2-allyl-2-isopropylacetamide rather than the drug itself were responsible for the

destruction of cytochrome *P*-450, both the increased destruction of the cytochrome observed in animals pretreated with phenobarbitone or DDT and the protective effect of compound SKF 525-A might reflect changes in the rate of metabolism of the drug.

Experiments were carried out *in vitro* to test this possibility. When liver microsomal fraction from phenobarbitone-treated rats was incubated with 2-allyl-2-isopropylacetamide a loss of cytochrome *P*-450 was observed only when NADPH, the cofactor essential for the metabolism of drugs by the liver microsomal fraction, was present. Some loss of cytochrome *P*-450 was also observed when the microsomal fraction was incubated in the presence of NADPH but with no 2-allyl-2-isopropylacetamide. Microsomal fraction incubated with 2-allyl-2-isopropylacetamide and NADPH had the characteristic brown-green discoloration at the end of the incubation, whereas microsomal fraction incubated with either alone did not (Table 5). These findings are compatible with the interpretation that 2-allyl-2-isopropylacetamide has to be metabolized in order to produce destruction of cytochrome *P*-450. However, since NADPH alone caused a loss of cytochrome *P*-450, another interpretation is also possible. A haem-oxidation system requiring NADPH and capable of producing (in the presence of cell sap) bilirubin from haem has been observed in liver microsomal fraction (Tenhunen, Marver & Schmid, 1968). It is possible that under the conditions of the present experiments *in vitro* the haem of cytochrome *P*-450 can also be degraded to bilirubin and that 2-allyl-2-isopropylacetamide in some way alters the mechanism of haem degradation and gives rise to 'green pigments' (see below). In this latter mechanism NADPH would be required for haem oxidation, whereas in the former it would be essential for metabolism of 2-allyl-2-isopropylacetamide to some reactive de-

Table 4. Effect of pretreatment with several drugs on the loss of cytochrome P-450 and on the degree of green discoloration of liver microsomal fraction caused by 2-allyl-2-isopropylacetamide in the rat

All rats were starved for 24 h before receiving saline (20 ml/kg body wt.) or 2-allyl-2-isopropylacetamide (400 mg/kg body wt.) subcutaneously. Some animals were treated with phenobarbitone or other drugs (before the administration of 2-allyl-2-isopropylacetamide) as indicated in the Materials and Methods section. The results are expressed as means $\pm$ s.e.m. of the numbers of observations given in parentheses. Probabilities of difference from corresponding saline-treated controls are indicated thus: ** $P < 0.01$; *** $P < 0.001$.									
Treatment before 2-allyl-2-isopropylacetamide	Time of killing (h) after 2-allyl-2- isopropylacetamide or saline	Treatment ...	Cytochrome P-450 content (nmol/g wet wt. of liver)		Loss of cytochrome P-450 due to 2-allyl-2-isopropyl- acetamide	Intensity of brown-green colour of microsomal fraction			
			Saline	2-Allyl-2-iso- propylacetamide					
None	1		22.1 $\pm$ 1.3 (4)	15.6 $\pm$ 1.0 (4)**	29.4	6.5	+		
	5		22.7 $\pm$ 1.5 (4)	9.6 $\pm$ 0.5 (4)***	57.7	13.1	++		
Phenobarbitone	1		64.3 $\pm$ 4.0 (6)	17.8 $\pm$ 0.7 (6)***	72.3	46.5	+++		
DDT	1		84.0 $\pm$ 4.6 (4)	29.3 $\pm$ 3.9 (4)***	65.1	54.7	+++		
Compound SKF 525-A	5		17.3 $\pm$ 2.9 (4)	15.1 $\pm$ 1.4 (4)	12.7	2.2	+		
	1		60.0 $\pm$ 2.7 (4)	26.4 $\pm$ 0.9 (4)**	56.0	33.6	++		
Phenobarbitone + compound SKF 525-A									

All rats were starved for 24h before receiving saline (20ml/kg body wt.) or 2-allyl-2-isopropylacetamide (400 mg/kg body wt.) subcutaneously. Some animals were treated with phenobarbitone or other drugs (before the administration of 2-allyl-2-isopropylacetamide) as indicated in the Materials and Methods section. The results are expressed as means±s.e.m. of the numbers of observations given in parentheses. Probabilities of difference from corresponding saline-treated controls are indicated thus: ** $P < 0.01$; *** $P < 0.001$.

Fig. 3. Effect of a single subcutaneous injection of 2-allyl-2-isopropylacetamide (400 mg/kg body wt.) on the concentrations of cytochrome P-450 in liver microsomal fraction of normal rats (○) and of phenobarbitone-induced rats (●). Each point represents the mean±s.e.m. of the logarithm of the value observed. The numbers of observations are given in parentheses.

rivative. So it is not possible to conclude with certainty from the results obtained either *in vivo* or *in vitro* whether 2-allyl-2-isopropylacetamide has to be metabolized in order to produce destruction of cytochrome P-450.

Loss of liver haem caused by 2-allyl-2-isopropylacetamide: conversion of haem into unidentified 'green pigments'. Schwartz & Ikeda (1955) described a discoloration of the liver of rats and rabbits given either 2-allyl-2-isopropylacetamide or the related drug Sedormid (2-isopropylpent-4-enoylurea), and they extracted and partially characterized certain 'green pigments' responsible for this abnormal colour. The significance of these pigments remained obscure. The present results strongly suggested that the abnormal pigments might arise from some chemical change in existing haem as a result of the action of 2-allyl-2-isopropylacetamide on the liver. Direct evidence for this was sought by prelabelling the liver haems of phenobarbitone-induced rats with radioactive 5-aminolaevulate before the administration of 2-allyl-2-isopropylacetamide and by studying the distribution of the label between haem and the fraction known from the work of Schwartz *et al.* (1960) to contain the 'green pigments'. The haem pools were labelled by giving 5-amino-[4-¹⁴C]laevulate 26h and [G-³H]5-aminolaevulate

Table 5. *Effect of incubation of rat liver microsomal fraction in the presence of 2-allyl-2-isopropylacetamide and/or NADPH on the concentration of cytochrome P-450*

The livers of two phenobarbitone-treated rats were perfused with ice-cold saline through the portal vein and homogenized in 1.15% (w/v) KCl, and the homogenates were pooled. The supernatant of a 9000g (20 min) centrifugation (equivalent to 400mg wet wt. of liver) was incubated in air at 37°C for 30 min with shaking (130 cycles/min) in 25ml conical flasks. The incubation mixture contained, in a total volume of 6ml, potassium phosphate buffer, pH 7.4 (500 μ mol), MgCl₂ (32 μ mol) and, where NADP⁺ was also present, glucose 6-phosphate (50 μ mol) and glucose 6-phosphate dehydrogenase (0.7 U). At the end of the incubation the samples were transferred to an ice bath and diluted with cold 1.15% KCl and the microsomal fraction was obtained by centrifugation. The results for cytochrome P-450 are the averages of two experiments with the individual results in parentheses.

Addition	Cytochrome P-450 recovered in microsomal fraction (% of that in sample not incubated)	Intensity of brown-green colour of microsomal fraction
None	87.7 (80.0, 95.4)	—
2-Allyl-2-isopropylacetamide (28.4 μ mol)	98.8 (88.9, 108.7)	—
2-Allyl-2-isopropylacetamide (28.4 μ mol)+NADP ⁺ (2 μ mol)	26.4 (23.3, 29.6)	+++
NADP ⁺ (2 μ mol)	61.7 (59.1, 64.4)	—

Table 6. *Recovery of ³H and ¹⁴C radioactivities in the total liver homogenate, in the total liver haem and in the 'green pigments' fraction of the liver from rats treated with 2-allyl-2-isopropylacetamide and from their controls after injection of 5-amino[4-¹⁴C]laevulate and [G-³H]5-aminolaevulate*

Phenobarbitone-induced rats were injected with 5-amino[4-¹⁴C]laevulate (1.5 μ Ci/rat) 26h and with [G-³H]5-aminolaevulate (8.7 μ Ci/rat) 2h before the administration of 2-allyl-2-isopropylacetamide. One group of animals was killed without further treatment at the time of 2-allyl-2-isopropylacetamide injection ('zero time'). Other rats were killed 1h after the injection of 2-allyl-2-isopropylacetamide or saline. Results are expressed as the means of three consistent observations.

Treatment	Recovery of radioactivity (nCi/total liver)					
	In liver homogenate		In total haem		In 'green pigments'	
	³ H	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C
None ('zero time')	1320	154	853	115	12.8	1.9
Saline (A)	1390	166	857	107	11.1	2.0
2-Allyl-2-isopropylacetamide (B)	1100	146	347	60.4	112.0	10.0
B/A ratio ...	0.79	0.88	0.40	0.56	10.09	5.0

2h before the drug. The times of injection of the labelled 5-aminolaevulates were chosen so that, when 2-allyl-2-isopropylacetamide was administered, ³H and ¹⁴C would be expected to be present in different proportions in haem pools of appreciably different rates of turnover.

Animals killed 1h after the administration of 2-allyl-2-isopropylacetamide showed only a relatively small decrease in the radioactivity recovered in the total liver homogenate (Table 6). A considerable loss of the radioactivity of both isotopes was found in the haem isolated from the liver homogenates of rats treated with 2-allyl-2-isopropylacetamide: the loss of ³H was greater than that of ¹⁴C, suggesting that rapidly-turning-over haem(s) might be preferentially affected. An increase in the

radioactivity of both isotopes was recovered in the 'green pigments' fraction, though this accounted for only a small portion of the radioactivity lost from haem (17 and 20% on average for ¹⁴C and ³H respectively). As noted for the radioactivity lost from haem, the gain of radioactivity in the 'green pigments' fraction also involved ³H to a greater extent than ¹⁴C. These findings afforded direct evidence for an increased destruction of liver haem after the administration of 2-allyl-2-isopropylacetamide and for the conversion of at least part of the lost haem into 'green pigments'.

In contrast with the results obtained by pre-labelling haem with [³H]5-aminolaevulate and 5-amino[¹⁴C]laevulate, when the liver haems were prelabelled with ⁵⁹Fe no increase was noted in the

Table 7. *Recovery of ^{59}Fe radioactivity in the total liver haem and in the 'green pigments' fraction of the liver from rats treated with 2-allyl-2-isopropylacetamide and from their controls after injection of ^{59}Fe ferrous ascorbate*

Phenobarbitone-induced rats were injected with ^{59}Fe ferrous ascorbate (13.9×10^6 c.p.m./kg body wt.) 2h before the administration of 2-allyl-2-isopropylacetamide. Rats were killed 1h after the injection of 2-allyl-2-isopropylacetamide or saline. The results are expressed as the means of two observations with the individual results in parentheses.

Treatment	Recovery of radioactivity (c.p.m./total liver)	
	In total haem	In 'green pigments'
Saline	35400 (35000, 35800)	59.5 (50, 69)
2-Allyl-2-isopropylacetamide	7750 (7200, 8300)	48.5 (41, 56)

radioactivity of the 'green pigments' fraction after the administration of 2-allyl-2-isopropylacetamide (Table 7), suggesting that these derivatives of haem (or at least that portion which is recovered after the complete isolation procedure) have lost most of their iron. This point requires further study, however, since the iron might have been present, but then lost during the isolation procedure.

The exact nature of these pigments and the mechanism by which they are produced from haem under the influence of 2-allyl-2-isopropylacetamide are not known. Recent results (G. Abbritti & F. De Matteis, unpublished work) obtained with several drugs chemically related to 2-allyl-2-isopropylacetamide strongly suggest the importance of the allyl group for the destruction of liver haem by these drugs. The way in which the allyl group is implicated is not known, although the results obtained *in vitro* in the presence of NADPH (see Table 5) suggest that the activity of the drug-metabolizing system of the liver microsomal fraction may be involved in this effect. It is possible that the drug (or its allyl group), as has already been discussed, needs to be converted by microsomal drug metabolism into an active agent that is in turn responsible for haem destruction.

Alternative mechanisms for the abnormal destruction of haem caused by 2-allyl-2-isopropylacetamide in the liver cannot be excluded. For example, it is possible that the structure of haem might be altered by peroxides, if these were produced during the metabolism of the drug, or that the drug might expose certain pools of haem (such as the cytochrome *P*-450 haem) to coupled oxidation by endogenously occurring reductants such as ascorbate or NADPH. This would give rise to several ill-defined pigments in a way similar to the ascorbate-catalysed breakdown of haem *in vitro* (Levin, 1966). Also, it would, by random cleavage of the four methine bridges of haem, give rise to a mixture of predominantly unphysiological bili-

verdin isomers, (Ó Carra & Colleran, 1969) that would not be converted into bilirubin (E. Colleran & P. Ó Carra, unpublished work cited by Ó Carra & Colleran, 1969). This mechanism would be compatible with the finding, in 2-allyl-2-isopropylacetamide-treated rats given $[2\text{-}^{14}\text{C}]\text{glycine}$, of a strong labelling of exhaled carbon monoxide (which might be expected to be produced from the cleavage of any of the four methine bridges of haem) with no corresponding increase in the labelling of bilirubin (Landaw, Callahan & Schmid, 1970). It would also be compatible with the heterogeneity of the 'green pigments' described by Schwartz & Ikeda (1955) after the administration of Sedormid: the free pigments or their methyl esters could be separated by paper or column chromatography into several components (some yellow and brown, others blue and green) reminiscent of the several coloured side products obtained *in vitro* on coupled oxidation of haemin either with hydrazine hydrate (Lemberg & Legge, 1949) or with ascorbate (Levin, 1966).

Possible relation of the loss of liver haem to the stimulation of 5-aminolaevulate synthetase activity caused by 2-allyl-2-isopropylacetamide. Since haem is known to exert a negative-feedback control on 5-aminolaevulate synthetase in bacterial (Burnham & Lascelles, 1963; Lascelles 1960), avian (Granick, 1966) and mammalian systems (Waxman, Collins & Tschudy, 1966), the possibility was considered (De Matteis, 1970) that destruction of liver haem might be implicated in the stimulation of 5-aminolaevulate synthetase activity and in the induction of porphyria by 2-allyl-2-isopropylacetamide. This possibility has already been considered by Marver, Kaufman & Manning (1968) on the basis of indirect evidence.

The following lines of evidence are in favour of this mechanism of stimulation of the enzyme activity: (1) changes in the cytochrome *P*-450 concentration and 5-aminolaevulate synthetase activity after a single dose of 2-allyl-2-isopropylacetamide behave

Table 8. *Effect of pretreatment with compound SKF 525-A or with phenobarbitone on the increase in 5-aminolaevulate synthetase activity caused by 2-allyl-2-isopropylacetamide and the relation of this latter effect to the loss of cytochrome P-450*

Rats were starved for 24h, then injected with saline (20 ml/kg body wt.) or 2-allyl-2-isopropylacetamide (400 mg/kg body wt.) subcutaneously and killed 5h later. Compound SKF 525-A and phenobarbitone were given as indicated in the Materials and Methods section. The results are expressed as means $\pm$ s.e.m. of the numbers of observations given in parentheses. Probabilities of difference from corresponding saline-treated controls are indicated thus: ** $P < 0.02$; *** $P < 0.001$.

Pretreatment	Treatment ...	5-Aminolaevulate synthetase activity (nmol/min per 100 mg of mitochondrial protein)		Increase in 5-aminolaevulate synthetase activity due to 2-allyl-2-isopropylacetamide (%)	Loss of cytochrome P-450 caused by 2-allyl-2-isopropylacetamide (nmol/g wet wt. of liver)
		Saline	2-Allyl-2-isopropylacetamide		
None		2.14 $\pm$ 0.24 (4)	5.33 $\pm$ 0.32 (4)***	149.0	13.1†
Compound SKF 525-A		1.73 $\pm$ 0.05 (4)	1.96 $\pm$ 0.14 (4)	13.0	2.2†
Phenobarbitone		3.08 $\pm$ 0.48 (7)	5.91 $\pm$ 0.84 (7)**	91.8	43.7†

† Consult Table 4 for the cytochrome P-450 concentrations observed in these animals.

‡ The cytochrome P-450 concentrations observed in these animals were as follows: saline-treated, 53.6 $\pm$ 4.0 (4) nmol/g wet wt. of liver; 2-allyl-2-isopropylacetamide-treated, 9.85 $\pm$ 0.55 (8) nmol/g wet wt. of liver.

in an approximately reciprocal fashion (De Matteis, 1970); (2) a loss of cytochrome P-450 is also observed in mice, rabbits or rats when the other porphyrogenic agents 3,5-diethoxycarbonyl-1,4-dihydrocollidine and griseofulvin are administered, despite the fact that they are not chemically related to 2-allyl-2-isopropylacetamide (Wada, Yano, Urata & Nakao, 1968; Waterfield, Del Favero & Gray, 1969; De Matteis, 1971); (3) within a group of drugs related to 2-allyl-2-isopropylacetamide there is a correlation between porphyrogenic activity after administration of a single dose of the drugs to the rat and the ability of the drugs to cause loss of cytochrome P-450 *in vivo* or green discoloration of rat liver microsomal fraction *in vitro* (G. Abbritti & F. De Matteis, unpublished work): both porphyria and microsomal damage appear to be related to the presence of an allyl group in the molecule of each of these drugs.

An attempt was made to obtain more conclusive evidence for this mechanism of stimulation of 5-aminolaevulate synthetase activity by relating the stimulation to the extent of liver haem loss (as judged from the cytochrome P-450 concentration) under conditions that affect the extent of the cytochrome P-450 loss caused by 2-allyl-2-isopropylacetamide (Table 8). It was found that pretreatment of rats with compound SKF 525-A, which largely prevented the loss of cytochrome P-450 caused by 2-allyl-2-isopropylacetamide, almost completely inhibited the stimulation of the enzyme activity. On the other hand pretreatment with phenobarbitone, which markedly increased the destruction of cytochrome P-450 by 2-allyl-2-isopropylacetamide, did not enhance the stimula-

tion of the enzyme activity as expected, but possibly decreased it slightly.

This finding does not necessarily exclude the possibility that 2-allyl-2-isopropylacetamide may stimulate 5-aminolaevulate synthetase activity by promoting destruction of liver haem. First, although the net loss of cytochrome P-450 was greatly increased by pretreatment with phenobarbitone, the absolute amounts of cytochrome P-450 that remained in the microsomal fraction, both at 1h and at 5h after the administration of 2-allyl-2-isopropylacetamide, were not lower in phenobarbitone-treated rats than in normal rats (see Fig. 3). Secondly, it is possible that destruction of another pool of liver haem may be more relevant to the stimulation of 5-aminolaevulate synthetase activity than is the destruction of cytochrome P-450: recent findings (F. De Matteis, unpublished work) indicate that, although the microsomal fraction is the main site of haem destruction by 2-allyl-2-isopropylacetamide, a loss of haem is also observed from the cell sap and from the mitochondrial fraction of the liver homogenate of treated animals.

Therefore, although the results of this present work and the other lines of evidence considered above suggest the possibility that destruction of liver haem may be implicated in the stimulation of 5-aminolaevulate synthetase activity by 2-allyl-2-isopropylacetamide, no definite conclusions about this can as yet be drawn.

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